

Release of Tumor Necrosis Factor Alpha and Interleukin 6 during Antibiotic Killing of *Escherichia coli* in Whole Blood: Influence of Antibiotic Class, Antibiotic Concentration, and Presence of Septic Serum

JAN M. PRINS,^{1*} ED J. KUIJPER,² MARCEL L. C. M. MEVISSSEN,³ PETER SPEELMAN,¹
AND SANDER J. H. VAN DEVENTER³

The Department of Internal Medicine, Division of Infectious Diseases, Tropical Medicine and AIDS,¹ the Department of Medical Microbiology,² and the Center for Hemostasis, Thrombosis, Atherosclerosis and Inflammation Research,³ Academic Medical Center, Amsterdam, The Netherlands

Received 31 October 1994/Returned for modification 16 February 1995/Accepted 26 March 1995

The concentration and accessibility of endotoxin can increase following antibiotic killing of gram-negative bacteria. There are indications that antibiotics may differ in this respect. We measured endotoxin levels in RPMI 1640 and tumor necrosis factor alpha (TNF- α) and interleukin-6 production in whole blood ex vivo after exposure of log-phase *Escherichia coli* to antibiotics belonging to different classes, in a final concentration of 0.5, 5, or 50 times the MIC. After 4 h of incubation at 50 times the MIC, ceftazidime and ciprofloxacin treatment resulted in levels of endotoxin, TNF- α , and interleukin-6 significantly higher than those of imipenem and gentamicin ($P < 0.001$). Similar differences in cytokine induction were measured after 8 h of incubation. At 0.5 times the MIC, the differences between the antibiotics in measured endotoxin and cytokine levels were small, with levels comparable to the levels in untreated cultures. Polymyxin B and, to a lesser degree, recombinant bactericidal/permeability-increasing protein 21 (rBPI-21) were found to be potent inhibitors of TNF- α release, supporting the concept that the differences between the antibiotics in cytokine production were indeed due to differences in amounts of biologically active endotoxin. The presence of serum from patients suffering from untreated sepsis decreased TNF- α production significantly, in a concentration-dependent manner.

Endotoxin, the lipopolysaccharide (LPS) constituent of the outer membrane of the cell wall of gram-negative bacteria, is considered to be the most important bacterial factor in the pathogenesis of the gram-negative septic syndrome. It induces the release of the cytokines tumor necrosis factor alpha (TNF- α) and interleukins 1 and 6 (IL-1 and IL-6). These cytokines activate a cascade of secondary inflammatory mediators, eventually leading to endothelial damage and hemodynamic and metabolic derangements (3). Whereas adequate antibiotic treatment is thought to be pivotal in the therapy of severe gram-negative infections, many in vitro as well as animal and clinical studies have indicated that the endotoxin concentration may increase following bacterial death caused by exposure to antibiotics (15, 26). Three mechanisms can account for this increase: (i) accumulation of bacterial biomass following antibiotic treatment, for instance because of filament formation (24, 25); (ii) an increase in the accessibility of endotoxin that remains bound to the bacteria, which was demonstrated even when sub-MIC levels of antibiotics were used (23); or (iii) release of unbound (free) endotoxin. To avoid confusion, the overall increase in the amount of biologically active endotoxin is best described as an increase in endotoxin exposure.

Because appropriate antibiotic treatment nevertheless significantly reduces mortality in sepsis (16), the question arises of whether equally effective antibiotics have a differential effect on the increase in endotoxin exposure following treatment. In

vitro, significant differences have been demonstrated between various β -lactam antibiotics in this respect. Imipenem and the aminoglycosides have only a modest endotoxin-exposing ability, whereas in most studies quinolones caused relatively large quantities of endotoxin to be exposed (26). These differences correlated with differences in cytokine production by isolated peripheral blood mononuclear cells or in whole blood ex vivo (1, 8, 27).

The contribution of each of the factors that can lead to an increase of endotoxin exposure and the effect of antibiotic concentration and class on antibiotic-induced endotoxin exposure and subsequent cytokine production have not been systematically investigated. Therefore, we studied endotoxin levels in culture medium and cytokine production in whole blood ex vivo after exposure of live *Escherichia coli* to increasing concentrations of antibiotics belonging to different antibiotic classes. To study whether differences in cytokine production were caused by the differences in endotoxin exposure, we investigated the effect of the addition of polymyxin B and bactericidal/permeability-increasing protein (BPI), both of which are known to neutralize endotoxin (6, 10). Finally, because the production of proinflammatory cytokines is downregulated in sepsis (5, 14, 21, 22, 30), we assessed the effect of septic serum on the differences in cytokine production during bacterial killing.

MATERIALS AND METHODS

Bacteria. *E. coli* O4:K2 was used in all experiments. This strain was originally isolated from a patient with bacterial meningitis. It was stored at -70°C and prior to use was subcultured on blood agar. One colony was suspended in 5 ml of RPMI 1640 (Gibco, Life Technologies Ltd., Paisley, Scotland), and the sus-

* Corresponding author. Mailing address: Room F4-222, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Phone: (020) 5669111. Fax: (020) 5664440.

pension was incubated overnight at 37°C. This suspension was diluted 100-fold the next morning in fresh RPMI 1640 medium and incubated at 37°C. When log-phase growth occurred, as indicated by continuously increasing density in a turbidity photometer (Trübungsphotometer LTP4; Dr. Lange, Düsseldorf, Germany), at a concentration of approximately 2×10^7 to 6×10^7 CFU/ml, the suspension was diluted 1,000-fold in prewarmed RPMI 1640, immediately prior to the start of the experiments.

Antibiotics. Stock solutions of ceftazidime (Glaxo, Zeist, the Netherlands), imipenem (Merck, Sharp and Dohme, Haarlem, the Netherlands), gentamicin (Schering-Plough, Amstelveen, the Netherlands), and ciprofloxacin (Bayer, Mijdrecht, the Netherlands) were prepared and stored according to the instructions of the manufacturers. The MICs for *E. coli* O4:K2 was determined in RPMI 1640 by standard dilution techniques. The MICs were 0.05 mg of ceftazidime, 0.08 mg of imipenem, 0.24 mg of gentamicin, and 0.004 mg of ciprofloxacin per liter.

Polymyxin B and rBPI-21. Polymyxin B (Pfizer, Capelle aan de Yssel, the Netherlands) was prepared according to the instructions of the manufacturer. *E. coli* O4:K2 was susceptible to polymyxin B, as assessed by a conventional disk diffusion assay. rBPI-21, a recombinant 21-kDa NH₂-terminal fragment of BPI, was kindly supplied by XOMA, Berkeley, Calif.

Septic serum. Serum from three patients with urosepsis, who had blood and urine cultures positive for *E. coli*, was obtained before initiation of antibiotic treatment. After collection of blood, serum was prepared by centrifugation at $1,500 \times g$ for 20 min at room temperature and was divided in aliquots, which were immediately frozen and stored at -20°C. From one aliquot, TNF- α and IL-6 levels were determined. The levels of TNF- α were 65, 153, and 142 pg/ml and the levels of IL-6 were 98, 2,151, and 125 pg/ml for the three patients, respectively. Total endotoxin levels in platelet-rich plasma samples, which were simultaneously obtained, were determined by a chromogenic *Limulus* amebocyte lysate assay (see below) to be <0.036, 0.060, and <0.036 EU/ml, respectively. After thawing for 15 min in 37°C, equal amounts of septic serum from the three patients were mixed. This pooled septic serum was used in the stimulation experiments. Nonseptic serum was collected from a healthy volunteer and processed under endotoxin-free conditions as described above.

Bacterial killing and endotoxin levels. Fifty microliters of the bacterial suspension (10^3 CFU) was added to pyrogen-free tubes (Falcon 2063; Becton Dickinson, Lincoln Park, N.J.) containing 900 μ l of prewarmed RPMI 1640; 50 μ l of an antibiotic solution was added to reach a final concentration of 0.5, 5, or 50 times the MIC. Control experiments were performed by addition of only 50 μ l of bacterial suspension or antibiotic solution. The tubes were subsequently incubated at 37°C for 4 h. All experiments were performed in triplicate.

For counting of viable bacterial numbers at the high antibiotic concentrations (5 and 50 times the MIC), 100- μ l aliquots were plated on Cled plates; at the low antibiotic concentrations and in the control experiments without antibiotics, 10- and 100-fold dilutions were made in phosphate-buffered saline and 10- μ l aliquots of the dilutions were plated on Cled plates. All countings were performed in duplicate.

For endotoxin measurements, the aliquots were immediately frozen after incubation and were stored at -20°C until assessment.

Morphological studies. *E. coli* O4:K2 (approximately 10^8 CFU/ml) was incubated in RPMI 1640 in the presence of the various antibiotics in final concentrations of 0.5 and 50 times the MIC. After 4 h, specimens were taken, brought on a slide, and fixed in a cacodylate-buffered (pH 7.4) glutaraldehyde-formaldehyde mixture for one night. The next morning, the slides were thoroughly rinsed in cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol, and dried at the critical point with CO₂. The slides were mounted on small metal plates with conducting carbon cement and were platinum coated. The specimens were studied in a Philips SEM 505 electron microscope, using the back scatter detector (B. Willekens, The Netherlands Ophthalmic Research Institute, Amsterdam, The Netherlands).

Whole blood stimulations. Blood was collected by venipuncture by using a pyrogen-free collecting system. Anticoagulation was obtained with pyrogen-free heparin (Organon Teknika BV; Bostel, the Netherlands; final concentration, 50 IE/ml of blood). The same donor was used in all experiments. Fifty microliters of the bacterial suspension (1.7×10^3 CFU) was added to pyrogen-free tubes (Falcon 2063) containing 900 μ l of heparinized whole blood; 50 μ l of an antibiotic solution was added to reach a final concentration of 0.5, 5, or 50 times the MIC. Control experiments were performed by addition of only 50 μ l of bacterial suspension or antibiotic solution. The tubes were incubated at 37°C for 4 or 8 h. Incubations were terminated by centrifugation ($1,500 \times g$ for 20 min at 4°C). The plasma was collected, immediately frozen, and stored at -20°C until assessments of TNF- α and IL-6. All experiments were performed in triplicate.

Effects of polymyxin B and rBPI-21. A 50- μ l bacterial suspension (approximately 3×10^3 CFU) and 50 μ l of an antibiotic solution (final concentration, 50 times the MIC) were added to 850 μ l of whole blood. A 50- μ l volume of polymyxin B (final concentration, 10 μ g/ml) or rBPI-21 (final concentration, 50 μ g/ml) was added either immediately or, in separate experiments, after 1 h of incubation.

Influence of septic serum. A 50- μ l bacterial suspension (approximately 2×10^3 CFU), 50 μ l of an antibiotic solution (final concentration, 50 times the MIC), and 0 (0%), 50 (5%), or 100 μ l (10%) of septic serum were added to 800 μ l of whole blood; to maintain equal volumes and to correct for the influence of additional serum, respectively, 100, 50, or 0 μ l of nonseptic serum was also added.

TABLE 1. Total endotoxin levels after 4 h of incubation of *E. coli* O4:K2 in RPMI 1640

MIC (fold)	Total endotoxin level (EU/ml) after 4 h of incubation in RPMI 1640 with the following ^a :				ANOVA
	Ceftazidime	Imipenem	Gentamicin	Ciprofloxacin	
50	10.3 \pm 0.5	0.7 \pm 0.2	0.4 \pm 0.1	1.8 \pm 0.6	<0.001 ^b
5	9.3 \pm 1.7	11.4 \pm 3.0	3.8 \pm 0	5.1 \pm 0.6	<0.01 ^c
0.5	110 \pm 37	70 \pm 22	68 \pm 16	55 \pm 7	0.09

^a Values are means \pm standard deviations of three experiments. Total endotoxin level at $t = 0$ h was 0.82 ± 0.03 ; after 4 h of incubation without antibiotics, the level was 80 ± 21 .

^b $P < 0.05$ (Newman-Keuls) for ceftazidime and ciprofloxacin versus imipenem and gentamicin and for ceftazidime versus ciprofloxacin.

^c $P < 0.05$ (Newman-Keuls) for ceftazidime and imipenem versus gentamicin and ciprofloxacin.

Assays. (i) Endotoxin assay. The endotoxin content was determined by a chromogenic *Limulus* amebocyte lysate assay; (Coatest Endotoxin; Chromogenix AB, Mölndal, Sweden). This method has a detection limit in blood of 0.036 EU/ml. Each sample was assayed in duplicate, and the results were expressed as the means.

(ii) TNF- α assay. TNF- α was measured by a sandwich TNF- α -enzyme-linked immunosorbent assay (ELISA) (11). The detection limit in plasma was 50 pg/ml. In the concentrations used, the antibiotics had no influence on TNF- α detection (data not shown).

(iii) IL-6 assay. IL-6 was assessed by an ELISA for human IL-6 with a baseline of 3 pg/ml, according to the instructions of the manufacturer (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands). With the concentrations used, the antibiotics had no influence on IL-6 detection (data not shown).

Statistical analysis. Values were expressed as means \pm standard deviations of three experiments. Data were analyzed using one-way analysis of variance (ANOVA). A P value of <0.05 was considered statistically significant. Multiple comparisons between groups were performed by the Newman-Keuls test.

RESULTS

Bacterial killing. In cultures without antibiotics, bacterial numbers increased 120-fold during 4 h of incubation, reaching 1.2×10^5 CFU/ml. At 5 and 50 times the MIC, bacterial counts were <10 CFU/ml after 4 h, and at 0.5 times the MIC, 4- to 12-fold growth occurred. As expected, the antibiotics did not differ in their ability to inhibit bacterial growth.

Bacterial morphology. Scanning electron microscopy revealed large differences in bacterial morphology following treatment with the antibiotics used (Fig. 1).

At 50 times the MIC, treatment with ceftazidime resulted in the formation of long (≤ 0.1 -mm) filaments. Treatment with ciprofloxacin also resulted in elongation of the bacteria, although it was less conspicuous than that after treatment with ceftazidime. In contrast, treatment with imipenem resulted in conversion into smaller, somewhat round bacteria, and gentamicin induced no remarkable morphological changes during treatment. At 0.5 times the MIC, the morphological changes were less dramatic, albeit comparable to the changes observed at 50 times the MIC.

Endotoxin levels. Endotoxin levels in RPMI 1640 supplemented with antibiotics alone were less than 0.060 EU/ml. The endotoxin level in RPMI 1640 after the addition of bacterial suspension at time (t) = 0 h was 0.82 ± 0.03 EU/ml. In untreated cultures, during 4 h endotoxin levels increased 100-fold, which is comparable to the increase in bacterial numbers. After 4 h at 50 times the MIC, ceftazidime and ciprofloxacin treatment induced significantly higher endotoxin levels than imipenem and gentamicin. Endotoxin levels after exposure to ceftazidime were, respectively, 14-, 25-, and 6-fold higher than the levels after exposure to imipenem, gentamicin, or ciprofloxacin (Table 1). At 5 times the MIC, the endotoxin levels

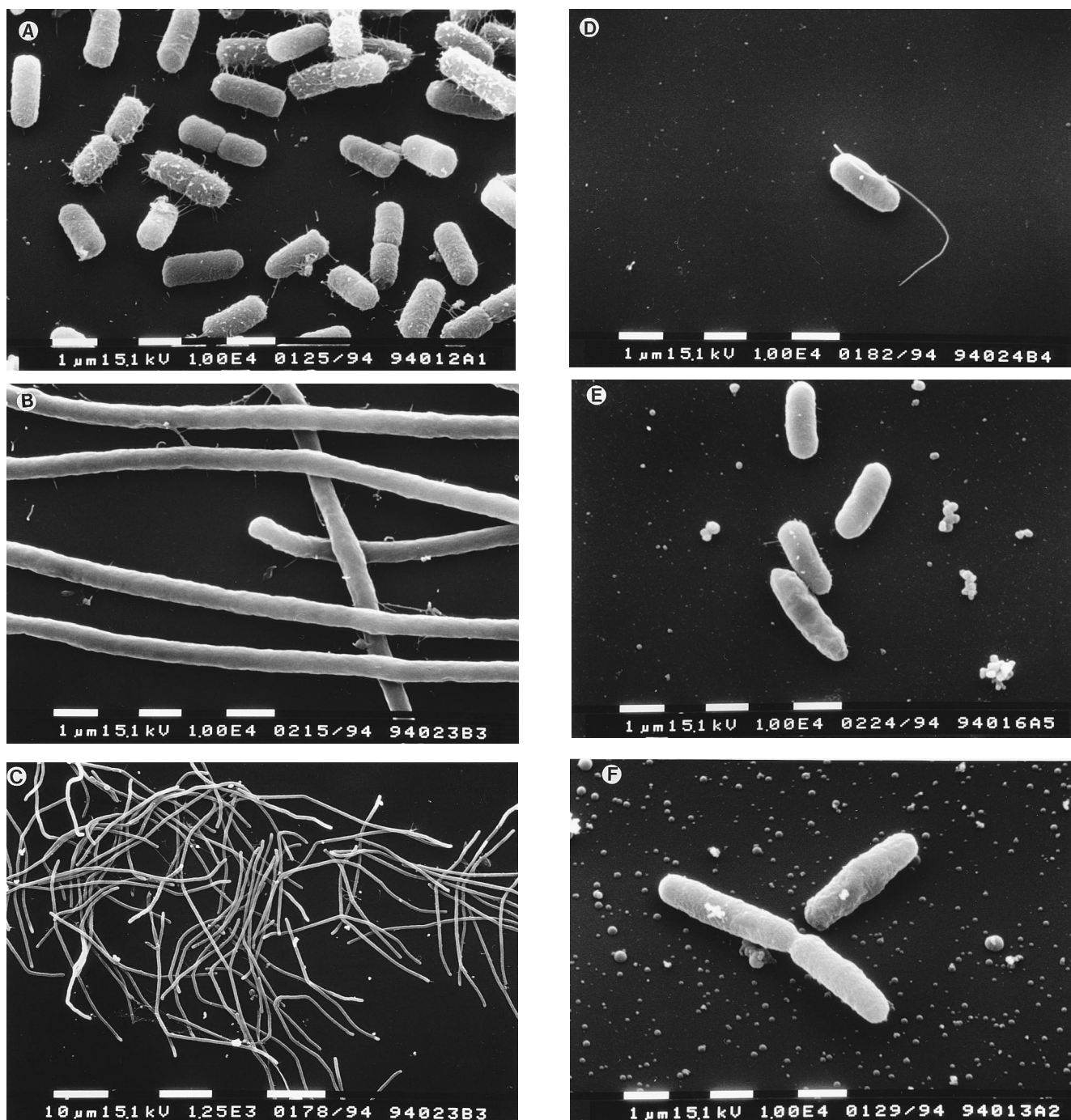


FIG. 1. Scanning electronmicrographs (magnification, $\times 7,000$) of *E. coli* O4:K2 after 4 h of incubation without antibiotics (A), with ceftazidime at magnifications of $\times 7,000$ (B) and $\times 875$ (C), imipenem (D), gentamicin (E), or ciprofloxacin (F), all in a concentration of 50 times the MIC; or with ceftazidime (G), imipenem (H), gentamicin (I), or ciprofloxacin (J) in a concentration of 0.5 times the MIC.

were higher, and gentamicin and ciprofloxacin showed the lowest levels. At 0.5 times the MIC, endotoxin levels were not significantly different for the four antibiotics or the untreated cultures.

TNF- α and IL-6 production in whole blood. TNF- α and IL-6 levels were below the detection limit after 4 h of incubation when only RPMI 1640 or antibiotics were added to whole blood. In the absence of antibiotics (untreated controls), the TNF- α concentration measured after 4 h incubation was 4,941

$\pm 1,044$ pg/ml. After 4 h at 50 times the MIC, the TNF- α levels were $7,678 \pm 1,093$ pg/ml for ceftazidime, $2,203 \pm 446$ pg/ml for imipenem, $3,173 \pm 248$ pg/ml for gentamicin, and $7,267 \pm 644$ pg/ml for ciprofloxacin. These differences in TNF- α induction between the antibiotics were highly significant ($P < 0.001$) (Fig. 2A). Also at 5 times the MIC, ceftazidime and ciprofloxacin induced significantly more TNF- α production than gentamicin ($P < 0.05$), while imipenem showed intermediate levels. At 0.5 times the MIC, TNF- α production was not significantly

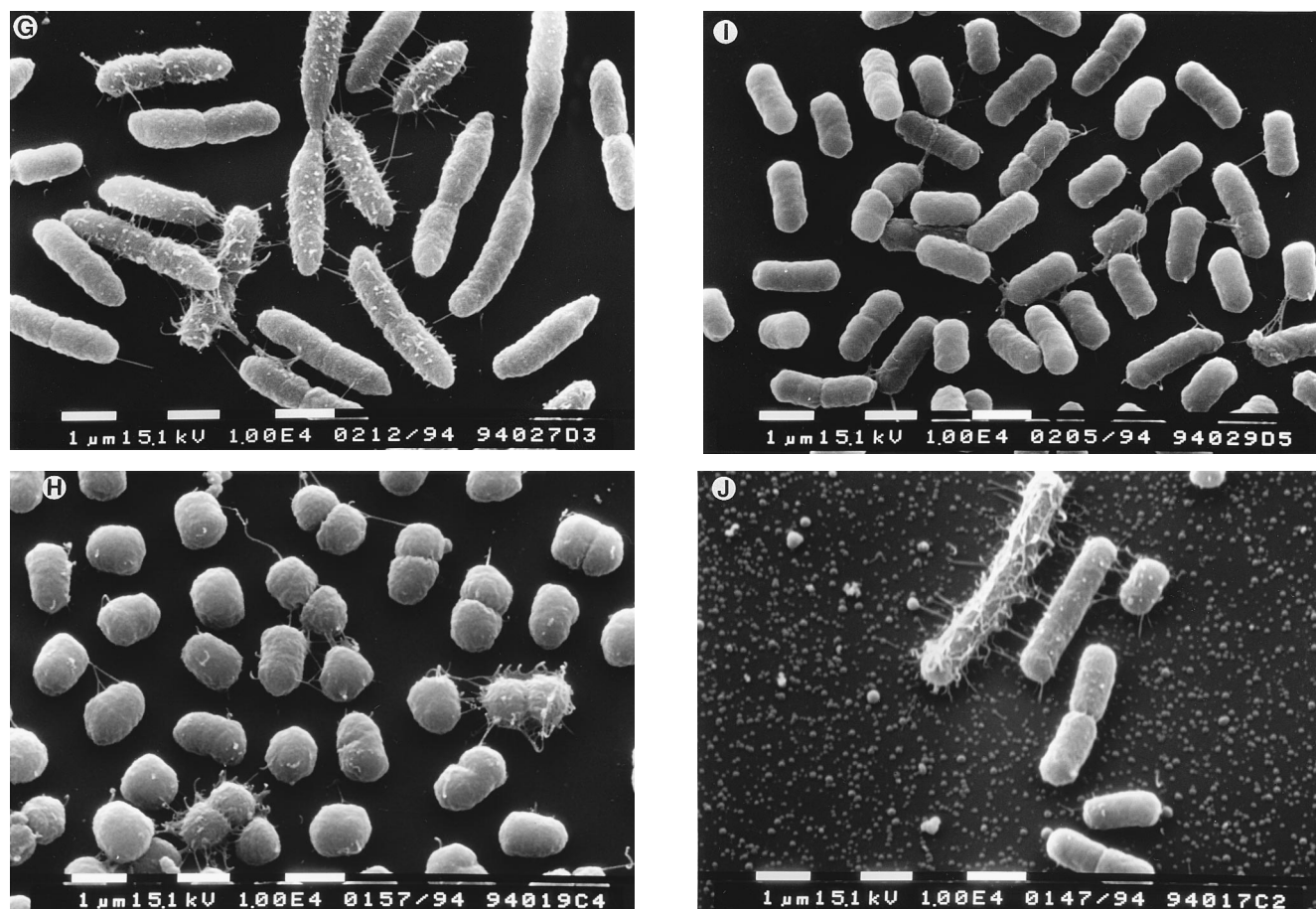


FIG. 1.—Continued.

different for the four antibiotics and was comparable to TNF- α production in untreated controls. At 50 times the MIC, similar differences between the antibiotics were observed with respect to IL-6 production (Fig. 2B). At 5 times the MIC, gentamicin induced significantly less IL-6 than all other antibiotics, and at 0.5 times the MIC, ceftazidime induced significantly more IL-6 than the other antibiotics or untreated cultures. The differential potency of the antibiotics to induce TNF- α or IL-6 was also observed at 8 h of incubation (Fig. 3).

Addition of polymyxin B and rBPI-21. Addition of 10 μ g of polymyxin B per ml at the start of the incubation period completely inhibited TNF- α production in whole blood (Fig. 4). When it was added after 1 h incubation, it still resulted in a protection level of greater than 90%. rBPI-21 in a concentration of 50 μ g/ml resulted in a 44 to 62% reduction in TNF- α production. Interestingly, when it was added after 1 h of incubation, the inhibition of TNF- α induction was only slightly less; however, for imipenem protection decreased to 72% of the TNF- α production without the addition of rBPI-21.

The viability of mononuclear cells after incubation with polymyxin B or rBPI-21 was established by eosin exclusion; viability was always greater than 99%.

Addition of septic serum. For all groups tested, addition of increasing percentages of septic serum resulted in a remarkable decline in TNF- α production (Fig. 5). Whereas with 5% septic serum ceftazidime and ciprofloxacin still induced significantly more TNF- α than imipenem or gentamicin, at 10% the differences between the antibiotics virtually disappeared. In

control experiments with only 5 or 10% of septic serum added but no bacteria, the TNF- α levels were below the detection limit.

DISCUSSION

The primary aim of this study was to investigate the influence of antibiotic class and concentration on endotoxin exposure in culture medium and cytokine production in whole blood ex vivo during antibiotic killing of *E. coli*. Whereas the efficacies of bacterial killing were comparable for the antibiotics at the three concentrations tested, important differences in bacterial morphology, endotoxin exposure, and cytokine production in whole blood were observed. Ciprofloxacin, and in particular ceftazidime, induced a remarkable elongation of bacteria, which is known as filamentation. Imipenem treatment resulted in the formation of small or round bacteria, and gentamicin induced no remarkable morphological changes. After 4 h of incubation at 50 times the MIC, ceftazidime and ciprofloxacin treatment resulted in significantly higher endotoxin, TNF- α , and IL-6 levels than those resulting from imipenem and gentamicin treatment. Similar differences in cytokine induction were measured after an 8-h incubation. At 0.5 times the MIC, the differences between the antibiotics in measured endotoxin and cytokine levels were small, with levels comparable to those in untreated cultures. At 5 times the MIC, results for endotoxin, TNF- α , and IL-6 were not uniform; however, in general, ceftazidime and gentamicin induced the

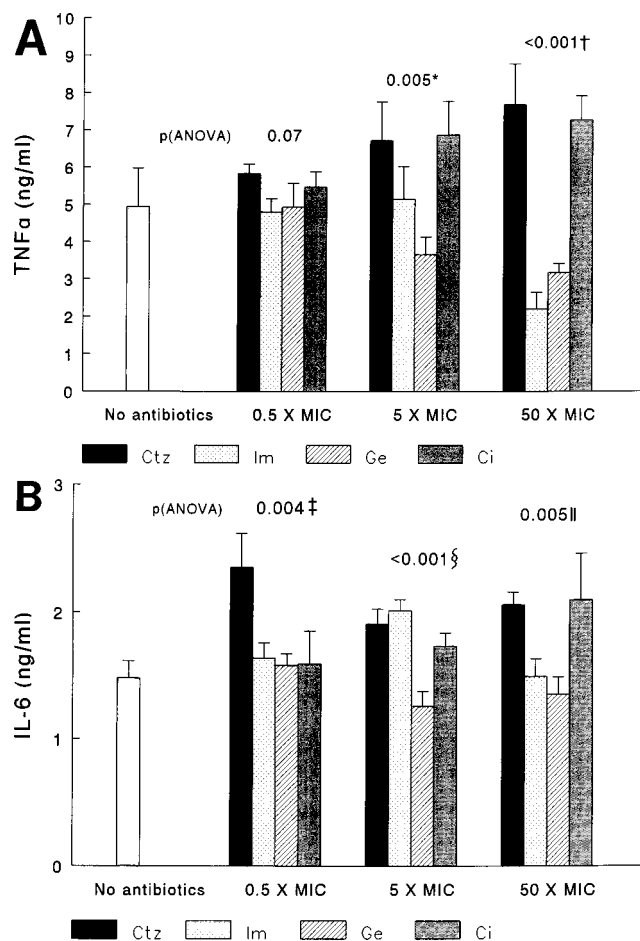


FIG. 2. TNF- α level (A) and IL-6 level (B) after 4 h of incubation of *E. coli* O4:K2 in whole blood. Ceftazidime (Ctz), imipenem (Im), gentamicin (Ge), and ciprofloxacin (Ci) were added in a final concentration of 0.5, 5, or 50 times the MIC. Treatment groups were compared by one-way ANOVA. Multiple comparisons between groups were performed by the Newman-Keuls test. *, $P < 0.05$ (Newman-Keuls) for ceftazidime versus imipenem, gentamicin, and ciprofloxacin; †, $P < 0.05$ (Newman-Keuls) for ceftazidime and ciprofloxacin versus imipenem and gentamicin; ‡, $P < 0.05$ (Newman-Keuls) for ceftazidime versus imipenem, gentamicin, and ciprofloxacin; §, $P < 0.05$ (Newman-Keuls) for ceftazidime, imipenem, and ciprofloxacin versus gentamicin and for imipenem versus ciprofloxacin; ||, $P < 0.05$ (Newman-Keuls) for ceftazidime and ciprofloxacin versus imipenem and gentamicin.

most and the least amounts of endotoxin and cytokines, respectively.

The observed differences between antibiotics in endotoxin-exposing potential can be in part ascribed to differences in accumulation of bacterial biomass following antibiotic treatment. This is substantiated by the results of the morphological studies. The antibiotics used are known to have different modes of antibacterial activity. Antibiotics with selective affinity for penicillin-binding protein 2 (PBP-2), such as imipenem and meropenem, cause conversion of the bacilli to round cells (spheroplasts), which is followed by loss of viability. This is not accompanied by extensive cell wall degradation (24, 28). In contrast, ceftazidime and other cephalosporins at lower concentrations have a high affinity for PBP-3. At higher concentrations these antibiotics also bind to PBP-1a. Inhibitors of PBP-3 cause selective inhibition of bacterial septation, which results in the formation of long filaments with a simultaneous increase in nonviable biomass (24, 25).

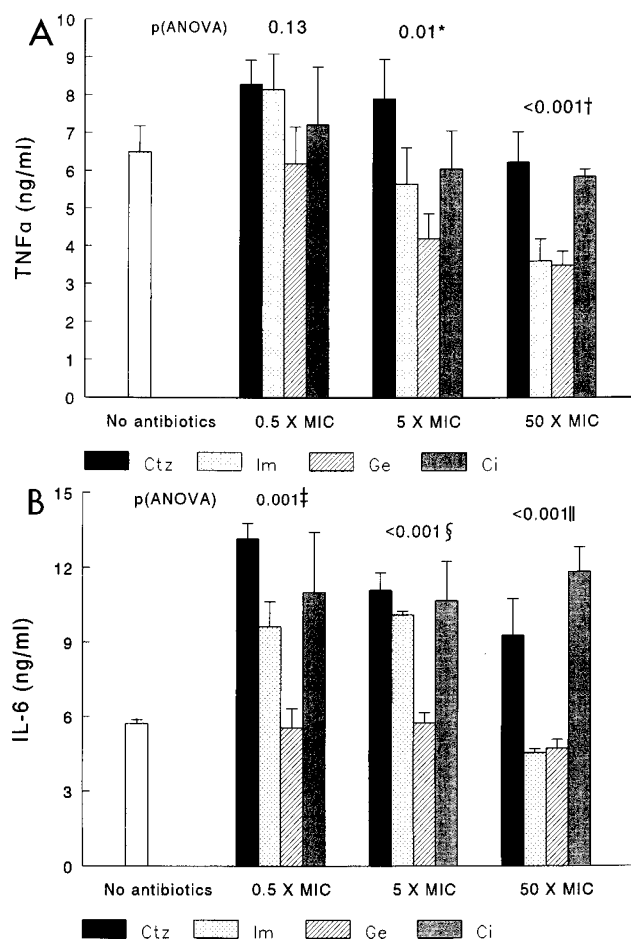


FIG. 3. TNF- α (A) and IL-6 (B) levels after 8 h of incubation of *E. coli* O4:K2 in whole blood. Ceftazidime (Ctz), imipenem (Im), gentamicin (Ge), and ciprofloxacin (Ci) were added in a final concentration of 0.5, 5, or 50 times the MIC. Treatment groups were compared by one-way ANOVA. *, $P < 0.05$ (Newman-Keuls) for ceftazidime versus imipenem, gentamicin, and ciprofloxacin; †, $P < 0.05$ (Newman-Keuls) for ceftazidime and ciprofloxacin versus imipenem and gentamicin; ‡, $P < 0.05$ (Newman-Keuls) for ceftazidime, imipenem, and ciprofloxacin versus gentamicin and for ceftazidime versus imipenem; §, $P < 0.05$ (Newman-Keuls) for ceftazidime, imipenem, and ciprofloxacin versus gentamicin; ||, $P < 0.05$ (Newman-Keuls) for ceftazidime and ciprofloxacin versus imipenem and gentamicin and for ciprofloxacin versus ceftazidime.

Although not primarily cell wall active, quinolones, which inhibit the enzyme DNA gyrase, induce morphological changes that are very similar to the effects of PBP-3-specific antibiotics, i.e., filamentation with increase of (nonviable) bacterial biomass (7, 29). Gentamicin and tobramycin treatment results in bacterial growth arrest and loss of viability in the absence of lysis or morphological changes (7, 29). It is likely that the small amount of endotoxin bioactivity induced by the aminoglycosides is partially explained by their ability to bind endotoxin (2, 13).

The presentation of LPS in an accessible form is important for detection in the *Limulus* assay, as well as for induction of cytokine production by monocytes. In viable bacteria, 98% of the endotoxin remains bound to the bacteria (9), and it has been demonstrated that bound endotoxin is 20 times less active in the *Limulus* assay than shed endotoxin (20). In our study, bacteria-bound and free endotoxin were probably equally active in the *Limulus* assay because of storage and the technique of measurement. This means that an increase in bacterial num-

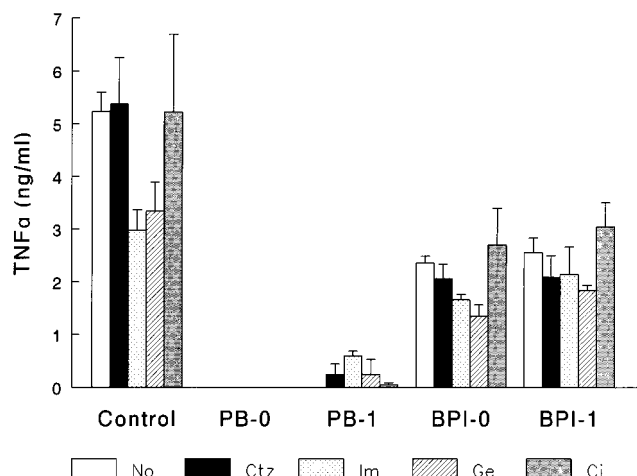


FIG. 4. TNF- α level after 4 h of incubation of *E. coli* O4:K2 in whole blood. No antibiotics were added (No), or ceftazidime (Ctz), imipenem (Im), gentamicin (Ge), or ciprofloxacin (Ci) were added in a final concentration of 50 times the MIC. A total of 10 μ g of polymyxin B (PB) or 50 μ g of rBPI-21 (BPI) per ml was added at $t = 0$ h (PB-0 and BPI-0) or after 1 h of incubation (PB-1 and BPI-1).

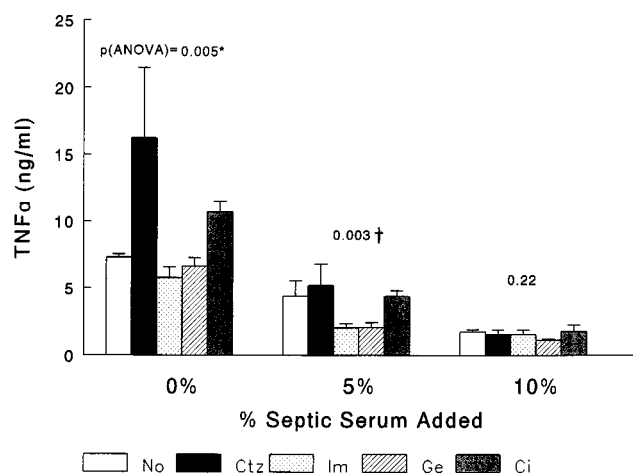


FIG. 5. TNF- α level after 4 h of incubation of *E. coli* O4:K2 in whole blood. No antibiotics were added (No), or ceftazidime (Ctz), imipenem (Im), gentamicin (Ge), or ciprofloxacin (Ci) was added in a final concentration of 50 times the MIC. Concentrations of 0, 5, or 10% (vol/vol) septic serum, and, respectively, 10, 5, or 0% (vol/vol) nonseptic serum was also added. Treatment groups were compared by one-way ANOVA. Multiple comparisons between groups were performed by the Newman-Keuls test. *, $P < 0.05$ (Newman-Keuls) for ceftazidime versus imipenem, gentamicin, and ciprofloxacin; †, $P < 0.05$ (Newman-Keuls) for ceftazidime and ciprofloxacin versus imipenem and gentamicin.

bers will give a proportionate increase in endotoxin concentration measured, which may explain why total endotoxin levels at 0.5 times the MIC and in untreated cultures were considerably higher than total endotoxin levels measured at 50 times the MIC, whereas the TNF- α and IL-6 levels were in the same order of magnitude for all antibiotic concentrations. At each antibiotic concentration, bacterial numbers were comparable for the four antibiotics, and at each concentration differences in endotoxin concentration paralleled differences in cytokine production. Following antibiotic treatment, even when antibiotics were used in sub-MIC concentrations, membrane-bound endotoxin becomes more accessible (23). This explains why no treatment resulted in endotoxin levels comparable to levels with treatment at 0.5 times the MIC, despite a 10-fold difference in bacterial numbers. Ampicillin and ciprofloxacin were shown to induce more enhancement of endotoxin accessibility than gentamicin (23). These observations all underscore the fact that in addition to bacterial biomass, the accessibility of (bacteria-bound) endotoxin plays a role in endotoxin exposure.

Finally, previous *in vitro* studies demonstrated that imipenem and the aminoglycosides induced less release of filtrable endotoxin during antibiotic killing of gram-negative bacteria than cephalosporins and the quinolones (26). Most cephalosporins, including ceftazidime, released less endotoxin at high concentrations than at low concentrations; however, at concentrations of up to 400 times the MIC, ceftazidime still induced more endotoxin than imipenem (8, 9). Differences in the amount of TNF- α produced by isolated mononuclear cells or human whole blood *ex vivo* after the addition of filtrates of bacterial cultures treated with different antibiotics have also been reported (1, 27).

It is not possible to distinguish the amount of biologically active cell-bound from free endotoxin in the *Limulus* amoebocyte lysate assay (or any other test), and, usually, both fractions are separated by filtration. However, measurement of LPS in cell-free medium (e.g., after filtration) would not account for the differences in bacterial biomass or differences in the accessibility of endotoxin. Therefore, the most appropriate model to evaluate differences between antibiotics in endotoxin exposure is measurement of cytokine release in whole blood following antibiotic treatment. In this model, a semilogarithmic relation

between the amount of biologically active endotoxin present and cytokine production has been established (4). In order to further characterize the importance of bioactive endotoxin for TNF- α induction, we investigated the effect of the addition of polymyxin B and rBPI. These proteins both bind endotoxin (6, 10) and specifically neutralize its cytokine-inducing effect (10, 27). Polymyxin B was found to be a potent inhibitor of TNF- α release, supporting the concept that the differences between the antibiotics in cytokine production were indeed due to differences in the amounts of biologically active endotoxin exposed. A direct toxic effect of polymyxin B on TNF- α -producing cells is not likely, since the viability of the mononuclear cells was not affected by polymyxin B, and the concentration used is in the range which can be measured in humans after an intravenous dose of 50 mg (17). Although rBPI is also known to bind endotoxin and to prevent TNF- α release induced by bacteria in whole blood (10, 31), rBPI-21 was less efficient in preventing cytokine production than polymyxin B. It is possible that the heparin used may have competed with endotoxin in binding rBPI (18). However, since in this model endotoxin concentration and TNF- α release are correlated in a semilogarithmic fashion (4), a 50% reduction in TNF- α release still indicates that approximately 90% of endotoxin must have been bound. When polymyxin B or rBPI was added 1 h of incubation, a significant protection was still offered. This underscores once more that rapid bacterial disintegration is not the main determinant of subsequent cytokine production.

The presence of septic serum decreased TNF- α production significantly in a concentration-dependent manner. Several studies demonstrated an impaired production of TNF- α , IL-1, and IL-6 after *ex vivo* endotoxin stimulation of whole blood or peripheral blood mononuclear cells from septic patients (14, 22, 30). This effect has been in part attributed to the anti-inflammatory cytokines IL-4 and IL-10 or elevated levels of glucocorticosteroids (21, 30). A reduction in the expression or alteration in the binding capacities of CD14, which serves as receptor on mononuclear cells for LPS binding protein (LBP)-LPS complexes, might be an alternative explanation; however,

data from earlier studies are conflicting on this point (12, 19). We have previously demonstrated that addition of septic serum to healthy peripheral blood mononuclear cells reduced TNF- α release (5). This could not solely be attributed to known inhibitors of the effects of endotoxin in vitro, including BPI, soluble CD14, or IL-10. The presence of other acute-phase reactants or elevated glucocorticosteroid levels in the septic serum might also be partially responsible. Hence, the cause of this impaired cytokine release in septic conditions is not exactly known. However, it follows from these observations and also from our study that in sepsis, the differences between antibiotics in endotoxin exposure may result in less-dramatic differences in the subsequent release of proinflammatory cytokines.

In conclusion, with clinically relevant concentrations, important differences were found between antibiotics belonging to different antibiotic classes in endotoxin exposure in culture media and in subsequent cytokine production in whole blood during killing of *E. coli*. The difference between antibiotics in endotoxin exposure can be ascribed to the specific mode of bacterial killing of the antibiotic and to differences in endotoxin accessibility and amount of free endotoxin released after treatment. Since it is likely that in septic conditions endotoxin-induced cytokine production is impaired, the question of whether these differences in endotoxin exposure have clinical relevance remains. This question can be resolved only by comparing these antibiotics in this respect under septic conditions.

ACKNOWLEDGMENT

S. J. H. van Deventer is a fellow of the Royal Dutch Academy of Arts and Sciences.

REFERENCES

1. Ardit, M., W. Kabat, and R. Yagov. 1993. Antibiotic-induced bacterial killing stimulates tumor necrosis factor- α release in whole blood. *J. Infect. Dis.* **167**:240–244.
2. Artenstein, A. W., and A. S. Cross. 1989. Inhibition of endotoxin reactivity by aminoglycosides. *J. Antimicrob. Chemother.* **24**:826–828.
3. Bone, R. C. 1991. The pathogenesis of sepsis. *Ann. Intern. Med.* **115**:457–469.
4. Bruin, K. F., M. A. M. von der Möhlen, B. H. F. Derkx, J. Jansen, J. W. ten Cate, and S. J. H. van Deventer. 1994. Characterization of the endotoxin-induced TNF release in whole blood and peripheral blood mononuclear cells, p. 31–42. In K. F. Bruin, Endotoxin responsiveness in humans. Ph.D. thesis, University of Amsterdam, Amsterdam.
5. Bruin, K. F., M. A. M. von der Möhlen, J. Jansen, M. H. Prins, J. W. ten Cate, and S. J. H. van Deventer. 1994. Septic serum inhibits endotoxin-induced tumor necrosis factor release by human peripheral blood mononuclear cells, p. 78–92. In K. F. Bruin, Endotoxin responsiveness in humans. Ph.D. thesis, University of Amsterdam, Amsterdam.
6. Cooperstock, M. S. 1974. Inactivation of endotoxin by polymyxin B. *Antimicrob. Agents Chemother.* **6**:422–425.
7. Crosby, H. A., J. F. Bion, C. W. Penn, and T. S. J. Elliott. 1994. Antibiotic-induced release of endotoxin from bacteria in vitro. *J. Med. Microbiol.* **40**:23–30.
8. Dofferhoff, A. S. M., M. T. Esselink, H. G. de Vries-Hospers, A. van Zanten, V. J. J. Bom, J. Weits, and E. Vellenga. 1993. The release of endotoxin from antibiotic-treated *Escherichia coli* and the production of tumour necrosis factor by human monocytes. *J. Antimicrob. Chemother.* **31**:373–384.
9. Dofferhoff, A. S. M., J. H. Nijland, H. G. de Vries-Hospers, P. O. M. Mulder, J. Weits, and V. J. J. Bom. 1991. Effects of different types and combinations of antimicrobial agents on endotoxin release from gram-negative bacteria: an in-vitro and in-vivo study. *Scand. J. Infect. Dis.* **23**:745–754.
10. Elsbach, P., and J. Weiss. 1993. The bactericidal/permeability-increasing protein (BPI), a potent element in host-defense against gram-negative bacteria and lipopolysaccharide. *Immunobiology* **187**:417–429.
11. Engelberts, L., A. Möller, G. J. M. Schoen, C. J. van der Linden, and W. A. Buurman. 1991. Evaluation of measurement of human TNF in plasma by ELISA. *Lymphokine Cytokine Res.* **10**:69–76.
12. Ertel, W., F. Krombach, J. P. Kremer, D. Jarrar, V. Thiele, J. Eymann, S. Muenzing, E. Faist, K. Messmer, and F. W. Schildberg. 1993. Mechanisms of cytokine cascade activation in patients with sepsis: normal cytokine transcription despite reduced CD14 receptor expression. *Surgery* **114**:243–251.
13. Focà, A., G. Matera, D. Iannello, M. C. Berlinghieri, and M. C. Liberto. 1991. Aminoglycosides modify the in vitro metachromatic reaction and murine generalized Shwartzman phenomenon induced by *Salmonella minnesota* R595 lipopolysaccharide. *Antimicrob. Agents Chemother.* **35**:2161–2164.
14. Helminen, M. 1991. Interleukin-1 production from peripheral blood monocytes in septic infections in children. *Scand. J. Infect. Dis.* **23**:607–611.
15. Hurley, J. C. 1992. Antibiotic-induced release of endotoxin: a reappraisal. *Clin. Infect. Dis.* **15**:840–854.
16. Kreger, B. E., D. E. Craven, and W. R. McCabe. 1980. Gram-negative bacteremia. IV. Re-evaluation of clinical features and treatment in 612 patients. *Am. J. Med.* **68**:344–355.
17. Kucers, A., and N. M. K. Bennett. 1987. The use of antibiotics, 4th ed., p. 899–913. William Heinemann Medical Books, London.
18. Little, R. G., D. N. Kelner, E. Lim, D. J. Burke, and P. J. Conlon. 1994. Functional domains of recombinant bactericidal/permeability increasing protein (rBPI₃₃). *J. Biol. Chem.* **269**:1865–1872.
19. Mathison, J., E. Wolfson, S. Steinemann, P. Tobias, and R. Ulevitch. 1993. Lipopolysaccharide (LPS) recognition in macrophages. Participation of LPS-binding protein and CD14 in LPS-induced adaptation in rabbit peritoneal exudate macrophages. *J. Clin. Invest.* **92**:2053–2059.
20. Mattsby-Baltzer, I., K. Lindgren, B. Lindholm, and L. Edebo. 1991. Endotoxin shedding by enterobacteria: free and cell-bound endotoxin differ in *Limulus* activity. *Infect. Immun.* **59**:689–695.
21. Mengozzi, M., and P. Ghezzi. 1993. Cytokine down-regulation in endotoxin tolerance. *Eur. Cytokine Netw.* **4**:89–98.
22. Munoz, C., J. Carlet, C. Fitting, B. Misset, J. P. Blériot, and J. M. Cavaillon. 1991. Dysregulation of in vitro cytokine production by monocytes during sepsis. *J. Clin. Invest.* **88**:1747–1754.
23. Nelson, D., T. E. S. Delahooke, and I. R. Poxton. 1993. Influence of subinhibitory levels of antibiotics on expression of *Escherichia coli* lipopolysaccharide and binding of anti-lipopolysaccharide monoclonal antibodies. *J. Med. Microbiol.* **39**:100–106.
24. Neu, H. C. 1983. Penicillin-binding proteins and role of amdinocillin in causing bacterial cell death. *Am. J. Med.* **75**(Suppl. 2A):9–20.
25. Neu, H. C. 1985. Relation of structural properties of beta-lactam antibiotics to antibacterial activity. *Am. J. Med.* **79**(Suppl. 2A):2–13.
26. Prins, J. M., S. J. H. van Deventer, E. J. Kuijper, and P. Speelman. 1994. Clinical relevance of antibiotic-induced endotoxin release. *Antimicrob. Agents Chemother.* **38**:1211–1218.
27. Simon, D. M., G. Koenig, and G. M. Trenholme. 1991. Differences in release of tumor necrosis factor from THP-1 cells stimulated by filtrates of antibiotic-killed *Escherichia coli*. *J. Infect. Dis.* **164**:800–802.
28. Tomasz, A. 1986. Penicillin-binding proteins and the antibacterial effectiveness of β -lactam antibiotics. *Rev. Infect. Dis.* **8**(Suppl. 3):S260–S278.
29. van den Berg, C., A. J. De Neeling, C. S. Schot, W. N. M. Hustinx, J. Wemer, and D. J. De Wildt. 1992. Delayed antibiotic-induced lysis of *Escherichia coli* in vitro is correlated with enhancement of LPS release. *Scand. J. Infect. Dis.* **24**:619–627.
30. Van Deuren, M., J. van der Ven-Jongekrijg, P. N. M. Demacker, A. K. M. Bartelink, R. van Dalen, R. W. Sauerwein, H. Gallati, J. L. Vannice, and J. W. M. van der Meer. 1994. Differential expression of proinflammatory cytokines and their inhibitors during the course of meningococcal infections. *J. Infect. Dis.* **169**:157–161.
31. Weiss, J., P. Elsbach, C. Shu, J. Castillo, L. Grinna, A. Horwitz, and G. Theofan. 1992. Human bactericidal/permeability-increasing protein and a recombinant NH₂-terminal fragment cause killing of serum-resistant gram-negative bacteria in whole blood and inhibit tumor necrosis factor release induced by the bacteria. *J. Clin. Invest.* **90**:1122–1130.